Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H_2 synthases

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Edited by John Vane, William Harvey Research Foundation, London, United Kingdom, and approved March 26, 2002 (received for review November 2, 2001)

Acetaminophen has antipyretic and analgesic properties yet differs from the nonsteroidal antiinflammatory drugs and inhibitors of prostaglandin H synthase (PGHS)-2 by exhibiting little effect on platelets or inflammation. We find parallel selectivity at a cellular level; acetaminophen inhibits PGHS activity with an IC_{50} of 4.3 μM in interleukin (IL)-1α-stimulated human umbilical vein endothelial cells, in contrast with an IC_{50} of 1,870 μM for the platelet, with 2 μM arachidonic acid as substrate. This difference is not caused by isoform selectivity, because acetaminophen inhibits purified ovine PGHS-1 and murine recombinant PGHS-2 equally. We explored the hypothesis that this difference in cellular responsiveness results from antagonism of the reductant action of acetaminophen on the PGHSs by cellular peroxides. Increasing the peroxide product of the PGHS-cyclooxygenase, prostaglandin G_2 (PGG_2), by elevating the concentration of either enzyme or substrate reverses the inhibitory action of acetaminophen, as does the addition of PGG_2 itself. 12-Hydroperoxyeicosatetraenoic acid (0.3 μM), a major product of the platelet, completely reverses the action of acetaminophen on PGHS-1. Inhibition of PGHS activity by acetaminophen in human umbilical vein endothelial cells is abrogated by *t*-butyl hydroperoxide. Together these findings support the hypothesis that the clinical action of acetaminophen is mediated by inhibition of PGHS activity, and that hydroperoxide concentration contributes to its cellular selectivity.

The pharmacological effects of acetaminophen (ApAP) are highly selective. It has antipyretic (1, 2) and moderate analgesic (3, 4) properties. However, unlike the nonsteroidal antiinflammatory drugs (NSAIDs) and inhibitors of prostaglandin H synthase (PGHS)-2, ApAP exhibits little or no antiinflammatory effect (5, 6). In most humans, its effect on platelets is slight at doses that are antipyretic (7, 8).

The first insight into the selectivity of ApAP derived from the findings of Flower and Vane (9) that the IC_{50} for inhibiting prostaglandin (PG)E_2 biosynthesis in the brain (93 μM) was 7-fold lower than that in the spleen. The potency of ApAP as an inhibitor of PG biosynthesis in the nervous system has been demonstrated in a number of investigations (10–14).

An additional locus of the selective action of ApAP was suggested by Green *et al.* (15), who demonstrated that ApAP inhibited prostacyclin biosynthesis in normal humans. ApAP, however, did not reduce the excretion of 2,3 dinor-thromboxane (Tx)B_2, a marker of the biosynthesis of TxA_2 in the platelet, consistent with its weak inhibition of platelet aggregation. This selective inhibition of prostacyclin biosynthesis was confirmed by O'Brien *et al.* (16). Prostacyclin biosynthesis occurs predominantly in the vessel wall in both the endothelium and smooth muscle (17–20). Using human umbilical vein endothelial cells (HUVECs) in culture, O'Brien *et al.* demonstrated inhibition by ApAP of prostacyclin biosynthesis (16). An endothelial site of action of ApAP is particularly germane to its antipyretic action in light of evidence that PGHS-2 is induced in brain endothelial cells in conjunction with fever (21–24).

The studies reported here have addressed the selective inhibition of prostacyclin biosynthesis by endothelial cells. The initial inquiry assessed whether this selectivity might result from inhibition of the prostacyclin synthase by ApAP. After finding that the action of ApAP in HUVECs is to inhibit the PGHSs, the determinants of the selectivity of this inhibition for endothelial cells were investigated. The basis for these investigations was derived from the accumulating evidence that ApAP inhibits the PGHSs by reducing the higher oxidation state of the enzymes (25–34). The PGHS-peroxidase, by reduction of a hydroperoxide to its alcohol, oxidizes the enzyme from its resting state (ferric heme) to the ferryloxo-protoporphyrin radical cation, which by intramolecular electron transfer generates the tyrosyl radical in the PGHS-cyclooxygenase site that is required for oxygenation of arachidonic acid (AA) (35–38). Thus it was hypothesized by Hanel and Lands (39) that peroxides, by oxidizing the enzyme to its catalytically active state, would oppose the action of drugs that reduce the oxidized form(s) of the enzyme back to the catalytically inactive resting state. They provided evidence for this hypothesis by demonstrating that lowering peroxide concentration with glutathione peroxidase enhances the inhibitory action of a number of reducing agents on PGHS-1, including ApAP; this finding has been extended recently to PGHS-2 (40). This hypothesis and its relevance to the cellular selectivity of ApAP have been examined in the investigations reported here.

Material and Methods

Materials. HUVECs were a generous gift from Douglas E. Vaughan. 12-Hydroperoxyeicosatetraenoic acid (HPETE), 12-hydroxyeicosatetraenoic acid (12-HETE), and PGG_2 were from Cayman Chemicals (Ann Arbor, MI). Medium 199, Hanks' Balanced Salt Solution (HBSS), penicillin, streptomycin, amphotericin B, ApAP, butylated hydroxyanisole, *t*-butyl hydroperoxide, hematin, phenol, Tris(hydroxymethyl)aminomethane (Tris), and IL-1α were from Sigma. [^{14}C]AA was from NEN. Silica gel 60A plates were from Whatman. PGHS-1 was purified from ram seminal vesicle as described (41). Wild-type murine PGHS-2 was expressed in SF-9 cells (Novagen) and purified as described (42). Sepharose 2B was from Amersham Pharmacia.

HUVECs. A freshly obtained umbilical cord was rinsed thoroughly with HBSS and then filled with a solution of collagenase 0.5 mg/ml in HBSS and incubated at 37°C for 10 min. Cells present in the collagenase solution were spun down at 100 $\times$ *g* for 5 min and resuspended in 10 ml of medium 199/15% FBS/25 μg/ml

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ApAP, acetaminophen; PGHS, prostaglandin H synthase; PG, prostaglandin; Tx, thromboxane; HUVEC, human umbilical vein endothelial cell; AA, arachidonic acid; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; NICI, negative ion chemical ionization mode.

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 www.pnas.org/cgi/doi/10.1073/pnas.102588199

endothelial growth mitogen/90 μg/ml heparin/100 units/ml penicillin/100 units/ml streptomycin/250 ng/ml amphotericin B (growth medium) and grown at 37°C in one 100-mm culture dish.

PG Synthesis in HUVECs. HUVECs grown to confluence in 6-well plates in growth medium were starved in medium 199/5% FBS/100 units/ml penicillin/100 units/ml streptomycin/250 ng/ml amphotericin B for 24 h. After activation with 1 ng/ml IL-1α for 24 h, the medium was changed to Hanks' solution/0.75% BSA, and ApAP was added at the indicated concentration for 20 min at 37°C. [$^2H_8$]AA then was added for 15 min, and the medium was harvested for GC/MS analysis.

Biosynthesis of [^{14}C]PGH$_2$. PGHS-1 (2,500 units, Oxford Biomedical Research, Oxford, MI) in 1 ml of 100 mM phosphate buffer, pH 7.5/500 μM phenol/25 μM hematin was preincubated at 37°C for 5 min. The reaction was started by adding 3.8 μCi (1 Ci = 37 GBq) of [^{14}C]AA (76 μM) and stopped after 2.5 min by adding 65 μl of 1 N HCl. PGs were extracted with 2 ml of ethyl acetate. The organic phase was dried with $\approx$0.5 mg of sodium sulfate, and the solvent was exchanged to hexane under argon. PGH$_2$ was purified on a CN-HPLC μBondapak CN, 3.9 $\times$ 300 mm, with an isocratic mobile phase consisting of hexane/isopropyl alcohol/acetic acid (100:3:0.02) at a flow rate of 1 ml/min. The radioactive fractions were pooled, dried under argon, exchanged to acetone, and stored at $-$70°C under argon in sealed vials until used.

Preparation of Washed Human Platelets. Human blood was obtained following a protocol approved by the Institutional Review Board of Vanderbilt University. Blood was drawn with a syringe containing 5 ml of 3.8% sodium citrate (final volume 50 ml) and then centrifuged in plastic tubes at 300 $\times$ *g* for 10 min at room temperature. The supernatant (platelet-rich plasma) was acidified to pH 6.4 with 0.15 M citric acid (43) and centrifuged at 1,000 $\times$ *g* for 10 min at room temperature. The pellet was resuspended with 5 ml of 24.4 mM sodium phosphate buffer, pH 6.5/0.113 M NaCl/5.5 mM glucose. After 15 min at room temperature, the platelets were purified on a Sepharose 2B column equilibrated with the same buffer. The eluted platelets were counted with a Coulter counter and diluted with 8.3 mM sodium phosphate buffer, pH 7.5/0.109 M NaCl/5.5 mM glucose for a final count of 300,000 platelets per μl.

Tx Synthesis in Platelets. Washed human platelets were incubated at room temperature in resuspension buffer. ApAP was added (final concentrations 0 μM, 66 μM, 200 μM, 400 μM, 1.3 mM, 2 mM, or 3.3 mM) for 20 min, and then AA was added (0.5, 2.0, or 20 μM). After 15 min at room temperature, TxB$_2$ was extracted with 400 μl of diethyl ether/methanol/4 M citric acid (30:4:1) and derivatized for analysis by GC/negative ion chemical ionization mode (NICI)/MS.

GC/MS Analysis. [$^2H_4$]Prostanoids (2 ng) were added to samples as internal standards. PGs were isolated and derivatized for analysis by GC/NICI/MS monitoring selected ions as described (44). The signals for the different molecules are: 6-keto-[$^2H_7$]PGF$_{1\alpha}$, m/z = 621; TxB$_2$, m/z = 614; and internal standards 6-keto-[$^2H_4$]PGF$_{1\alpha}$, m/z = 618; [$^2H_4$]TxB$_2$, m/z = 618; [$^2H_4$]PGE$_2$, m/z = 528. To account for the deuterium–protium exchange at the position C12 of [$^2H_7$]PGE$_2$, the summation of the signals obtained at m/z = 530, 531, and 532 was performed (45).

PGHS-1 and PGHS-2 Incubation with [^{14}C]AA. Ovine PGHS-1 or wild-type murine PGHS-2 (specific activity, 202 and 109 mol of AA per min per mol of enzyme, respectively) was preincubated on ice for 20 min with 2 molar equivalents of hematin in Tris·HCl buffer, pH 8.0/500 μM phenol. This solution then was warmed for 5 min at 37°C in the presence or absence of ApAP (final concentrations, 0.3, 0.5, 1, and 3 mM). [^{14}C]AA, 4.8 nCi (0.5 μM final concentration), in Tris·HCl buffer, pH 8.0, was preincubated at 37°C for 2 min. The reaction was initiated by adding PGHS-2 (10 nM final concentration) or PGHS-1 (5.4 nM final concentration) to a total volume of 200 μl and terminated after 8 sec by the addition of 200 μl of ice-cold diethyl ether/methanol/4 M citric acid (30:4:1) containing 8 μg of butylated hydroxyanisole as antioxidant and 8 μg of unlabeled AA as a carrier. The organic layer was loaded on a silica plate and eluted with the organic phase of ethyl acetate/isooctane/water/glacial acetic acid (45:25:50:1). TLC plates were analyzed for radioactivity by a Bioscan (Washington, DC) AR-2000 imaging scanner. Graphical analysis was performed with WIN-SCAN software (Bioscan). PGHS-1 and PGHS-2 activity was expressed as activity relative to control (no ApAP added). Activity was determined as pmol of AA converted to product·min·pmol of enzyme.

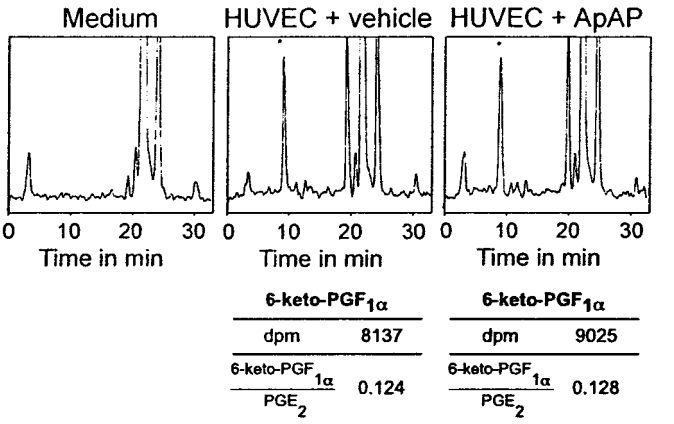

Fig. 1. ApAP does not inhibit prostacyclin synthase activity in HUVECs. HUVECs were grown in the presence of 15% FBS until confluence, medium was exchanged with 0.75% HBSS/BSA, and ApAP (final concentration 330 μM) or ethanol vehicle was added. After 2 h at 37°C, the medium was replaced with the same medium, [^{14}C]PGH$_2$, added for 15 min, and the medium was analyzed by reversed-phase HPLC coupled to a radioactive detector. As a control, [^{14}C]PGH$_2$ was added to the medium without HUVECs. 6-Keto-PGF$_{1\alpha}$ is indicated by a star. The results are expressed as the ratio of 6-keto-PGF$_{1\alpha}$ to PGE$_2$ (the rearrangement product of PGH$_2$).

Addition of 12-HPETE, 12-HETE, or PGG$_2$. As needed, 7–10 μl of hydroperoxides or alcohol were added to the 20-μl solution containing [^{14}C]AA immediately before initiating the reaction with PGHS. 12-HPETE and 12-HETE were in solution in Tris·HCl buffer, pH 8.0. PGG$_2$ was in dry acetone. Tris·HCl buffer, pH 8.0, or acetone was added to control samples.

Statistical Analysis. The statistical significance of differences was analyzed by the analysis of variance with subsequent Tukey's *t* or Student's *t* tests when comparing two series of data.

Results

The Effect of ApAP on Prostacyclin Synthase Activity in HUVECs. ApAP had no effect on conversion of [^{14}C]PGH$_2$ to 6-keto-[^{14}C]PGF$_{1\alpha}$ in HUVECs (Fig. 1). Although 330 μM ApAP did not inhibit the activity of prostacyclin synthase, it blocked the formation of endogenous (unlabeled) prostacyclin (GC/MS analysis of 6-keto-PGF$_{1\alpha}$) by 68%.

The Effect of ApAP on Prostacyclin Synthesis in HUVECs Stimulated with IL-1α. Both 6-keto-PGF$_{1\alpha}$ and PGE$_2$ were measured in HUVECs after the addition of 20 μM AA. When PGHS-2 was

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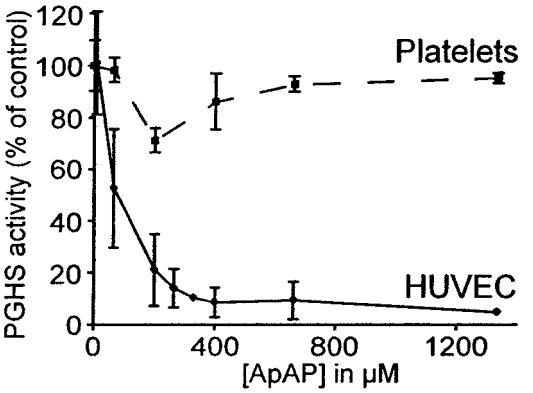

Fig. 2. Comparison of inhibition by ApAP of PGHS activity in HUVECs and platelets. Synthesis of TxB_2 in washed human platelets and of 6-keto-$PGF_{1\alpha}$ in HUVECs was assayed in the presence of increasing concentrations of ApAP (*Material and Methods*). AA (20 μM) was added as substrate. The prostanoid concentration present in the medium was determined by GC/NICI/MS and normalized to the value of the control (no ApAP was added) for each cell type. The data points represent the average of at least three independent experiments, each performed in triplicate, ± SD.

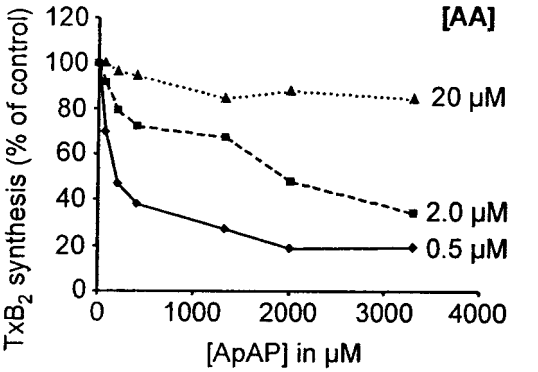

Fig. 3. Effect of the concentration of AA on the inhibition by ApAP of TxB_2 synthesis in human platelets. Washed human platelets were incubated at room temperature with ApAP at the indicated concentrations. After 20 min, AA was added (final concentrations, 0.5, 2.0, and 20 μM). After 15 min, TxB_2 was extracted with 400 μl of diethyl ether/methanol/4 M citric acid (30:4:1) and derivatized for analysis by GC/NICI/MS. The concentration of TxB_2 present in the medium is represented as a percentage of the control to which no ApAP was added. Experiments were performed in triplicate.

induced by IL-1α (46), prostacyclin biosynthesis increased by 13-fold, and PGE_2 formation was elevated by 70-fold. The IC_{50} of ApAP for prostacyclin biosynthesis is 72 μM at this substrate concentration, and there is a corresponding inhibition of PGE_2 formation (IC_{50} = 57 μM).

Differential Inhibition of PGHSs in HUVECs and Human Platelets. Using 20 μM AA as substrate, we observed that ApAP had no effect on platelet TxA_2 biosynthesis at concentrations up to 1,320 μM, contrasting with the almost complete inhibition of PGHS activity in HUVECs (Fig. 2). This marked difference could not result from differential inhibition of the PGHS isoforms, because ApAP is equipotent as an inhibitor of the activities of ovine PGHS-1 (IC_{50} = 419 μM) and murine PGHS-2 (IC_{50} = 372 μM).

The Effect of the Concentration of AA on ApAP Action in Cells. We contrasted the finding of an IC_{50} of 105 μM for inhibition of TxB_2 formation in clotting blood by ApAP (47) with our finding that 1,320 μM ApAP did not block TxB_2 production in platelets incubated with 20 μM AA. We hypothesized that these differences could reflect antagonism of ApAP action by the high levels of PGG_2 generated from the high concentration of AA used. Accordingly, the concentration of AA markedly altered the effect of ApAP (Fig. 3). The IC_{50} for ApAP at 0.5 μM AA was 210 μM and increased to 1,870 μM at 2 μM AA. At a substrate concentration of 20 μM, the IC_{50} exceeded 3,000 μM. In thrombin-activated platelets, the IC_{50} for ApAP was 120 μM.

Reducing the concentration of substrate also increased the inhibitory potency of ApAP on prostacyclin biosynthesis in IL-1α-stimulated HUVECs (Fig. 4). With 2 μM AA, the IC_{50} of ApAP is only 4.3 μM. Moreover, at this concentration of substrate, ApAP is far more potent as an inhibitor of prostanoid biosynthesis in the endothelial cells than in the platelets.

Enzyme and Substrate Concentrations as Determinants of ApAP Action on Purified PGHSs. Raising the concentration of PGHS-2 from 10 to 100 nM significantly attenuated the inhibitory action of ApAP (Fig. 5*A*). Under the same experimental conditions, the dependence of ApAP on substrate concentration was confirmed (Fig. 5*B*). Accordingly, when high concentrations of both enzyme and substrate were used, the inhibitory action of ApAP was reversed profoundly; 3 mM ApAP did not inhibit the activity of 200 nM PGHS-2 incubated with 50 μM AA, compared with an IC_{50} of 372 μM for ApAP when 10 nM of enzyme and 0.5 μM AA were used (data not shown).

The Effect of Peroxide Concentration on the Inhibition of Prostacyclin Biosynthesis in HUVECs. Pretreatment of HUVECs with *t*-butyl hydroperoxide alone in concentrations up to 200 μM had little effect on prostacyclin biosynthesis. However, the almost complete inhibition of prostacyclin production by 660 μM ApAP was reversed by *t*-butyl hydroperoxide in a concentration-dependent manner, such that 150 μM *t*-butyl hydroperoxide totally abolished the inhibitory action of ApAP (Fig. 6).

Lipid Peroxides Antagonize the Action of ApAP on Purified PGHSs. PGG_2 alone stimulated the catalytic activity of PGHS-1 (Table 1) and significantly attenuated the inhibitory action of ApAP on PGHS-1 (Fig. 7).

12-HPETE was found to enhance PGHS-1 activity, whereas it reduced PGHS-2 activity at concentrations above 0.2 μM, which is consistent with the evidence indicating a greater efficiency of

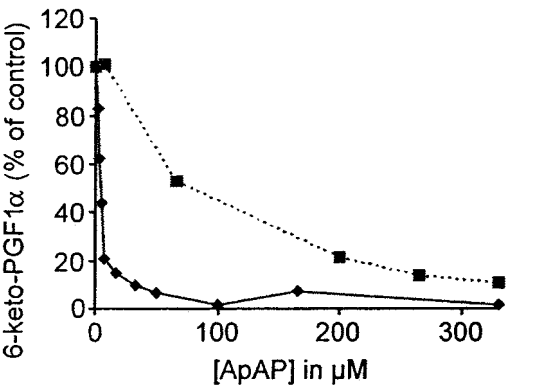

Fig. 4. Effect of the concentration of AA on the inhibition by ApAP of 6-keto-$PGF_{1\alpha}$ synthesis by IL-1α-activated HUVECs. HUVECs were starved for 24 h in the presence of 5% FBS before activation for 24 h with 1 ng/ml IL-1α. The medium was exchanged with 0.75% HBSS/BSA and ApAP at the concentrations indicated. After 20 min at 37°C, 2 (♦) or 20 (⋆) μM AA was added for 15 min, and the medium was collected for analysis by GC/NICI/MS. The concentrations of 6-keto-$PGF_{1\alpha}$ present in the medium are represented as a percentage of the control to which no ApAP was added. 100% represents 3.5 ng of 6-keto-$PGF_{1\alpha}$ per ml of medium.

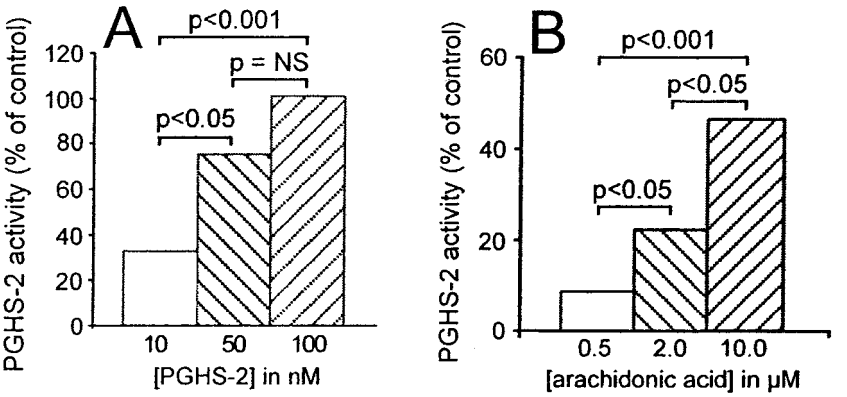

Fig. 5. Effect of the concentration of enzyme and substrate on the inhibition of PGHS-2 by ApAP. PGHS-2 was reconstituted (*Material and Methods*). The reaction was started by adding [^{14}C]AA and stopped after 8 sec. The data points represent the average of triplicates. NS, not significant. (*A*) PGHS-2 at the concentrations indicated was preincubated at 37°C with 1 mM ApAP. [^{14}C]AA was added (final concentration, 10 μM). (*B*) PGHS-2 (10 nM) was preincubated at 37°C with 1 mM ApAP. [^{14}C]AA was added at the concentrations indicated. PGHS-2 activity is expressed as a percentage of the control to which no ApAP was added.

hydroperoxide activation in PGHS-2 than in PGHS-1 (48, 49) that is associated with a difference in redox coupling between the heme center and tyrosyl radicals in the two isoforms (50). The inhibitory effect of ApAP (1 mM) on PGHS-1 is reversed totally by 12-HPETE at concentrations below that of substrate (Fig. 8). The action of ApAP on PGHS-2 also was attenuated by 12-HPETE (Fig. 8). To confirm the importance of the hydroperoxide we performed the same experiments using 12-HETE, the reduced form of 12-HPETE. The alcohol was found to have no effect on the inhibition of PGHS-1 by ApAP (data not shown).

Discussion

ApAP inhibits prostacyclin biosynthesis in HUVECs by blocking PGHS activity without inhibiting the biotransformation of PGH_2 to prostacyclin. The concomitant inhibition of the production of both PGE_2 and prostacyclin provides further evidence that ApAP acts at a step proximal to the prostacyclin synthase. Provision of exogenous AA as substrate for the PGHSs excludes an action of ApAP on the release of AA by phospholipases. Based on these findings, it is highly probable that inhibition of prostacyclin biosynthesis by ApAP *in vivo* (15, 16) also results from inhibition of PGHS activity.

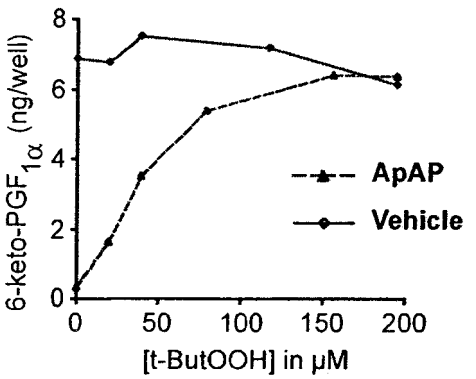

Fig. 6. Effect of *t*-butyl hydroperoxide (t-ButOOH) on the inhibition by ApAP of 6-keto-$PGF_{1\alpha}$ synthesis by IL-1α-activated HUVECs. HUVECs were starved for 24 h in the presence of 5% FBS before activation for 24 h with 1 ng/ml IL-1α. The medium was exchanged with 0.75% HBSS/BSA, and 666 μM ApAP or vehicle was added. After 20 min at 37°C, *t*-butyl hydroperoxide was added at the indicated concentration, immediately followed by AA. After 15 min, the medium was collected for analysis of 6-keto-$PGF_{1\alpha}$.

In contrast with endothelial cells, ApAP is much less potent as an inhibitor of PGHS activity in platelets and does not block TxA_2 biosynthesis *in vivo* (15, 16). Although PGHS-2 is the predominant PGHS isoform in IL-1α-stimulated HUVECs and PGHS-1 is the isoform in platelets, we found that the selectivity of ApAP for the HUVECs cannot be explained by a greater inhibitory action on PGHS-2. The possible contribution of peroxide concentration to this cellular selectivity (39) then was examined. Inhibition of prostacyclin biosynthesis by ApAP in IL-1α-stimulated HUVECs was reversed completely by the addition of *t*-butyl hydroperoxide to the cells, supporting the concept that intracellular peroxide levels influence the action of ApAP. After platelet activation, a substantial amount of 12-HPETE is formed via the platelet 12-lipoxygenase (51), and it is an activator of PGHS-1 in these cells (52). Accordingly, 12-HPETE antagonized the action of ApAP on both PGHS isoforms and did so at concentrations less than that of the substrate. To demonstrate further the specificity of the hydroperoxide moiety in this process, we show that 12-HETE, the reduced form of 12-HPETE, is unable to antagonize ApAP inhibition of PGHS-1. Accordingly, biosynthesis of 12-HPETE by platelets is likely to antagonize the effect of ApAP in this cell, which is relatively resistant to the effects of ApAP.

The effect of peroxide concentration on the action of ApAP is consistent with the concept of PGHS isoforms as bifunctional enzymes operating in a branched chain mechanism in which the tyrosyl radical in the PGHS-cyclooxygenase site is required for the cyclooxygenase activity (36, 53). Activation of the enzyme results from reduction of a peroxide at the PGHS-peroxidase site, generating a higher oxidative state of the heme, the ferryloxo protoporphyrin radical cation, that through intramo-

Table 1. The PGHS-peroxidase substrate, PGG_2, increases the oxygenation of AA by PGHS-1

[PGG_2], μM	0.0	0.25	0.5	1.0	7.0
PGHS-1 activity	100.0	168.4	172.9	198.7	256.1

PGHS-1 was reconstituted (*Material and Methods*). PGG_2 in solution in acetone was added at the concentrations indicated, immediately followed by [^{14}C]AA in solution in Tris·HCl buffer (0.5 μM final concentration). PGHS-1 activity was assessed by determination of the conversion of [^{14}C]AA to the oxygenated product by utilizing analysis by TLC (*Material and Methods*). The PGHS-1 activity is expressed as a percentage of the control to which no PGG_2 was added. The data points represent the average of at least three experiments.

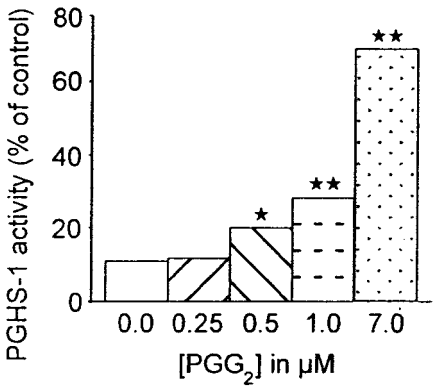

Fig. 7. Effect of PGG_2 concentration on inhibition of PGHS-1 by ApAP. PGHS-1 was reconstituted (*Material and Methods*) in the presence of 0.5 mM ApAP. PGG_2 was added at the concentrations indicated, immediately followed by [^{14}C]AA (final concentration, 0.5 μM). The reaction was stopped after 8 sec. PGHS activity is expressed as a percentage of the control to which no ApAP was added. The significance of differences between each concentration of substrate and the control was determined ($n = 4$: *, $P < 0.01$; **, $P < 0.001$).

lecular electron transfer yields the tyrosyl radical. The tyrosyl radical is reduced in the process of forming the AA radical and is regenerated by reduction of the PGG_2 radical to PGG_2. There are several points at which ApAP could act as a reductant to halt PGHS-cyclooxygenase activity. Reduction of the protoporphyrin radical cation would prevent the formation of the tyrosyl radical. There is abundant evidence that ApAP and other reductants act to reduce this higher oxidative state of the PGHS-peroxidase and other peroxidases to the ferric or "resting" state, a process in which ApAP serves as a cosubstrate for the peroxidases (25–34). By contrast, ibuprofen, indomethacin, and flurbiprofen, inhibitors that block access of substrate to the PGHS-cyclooxygenase catalytic site, do not act as cosubstrates of the PGHS-peroxidase (28). It also has been proposed that ApAP could directly reduce the tyrosyl radical or prevent its regeneration by reducing the PGG_2 radical (27, 54). It follows that inhibition of PGHSs by a reductant action of ApAP at any of these sites could be antagonized by a peroxide that returns the enzyme to its higher oxidation state.

We find that the hydroperoxides, 12-HPETE and PGG_2, enhance the activity of PGHS-1 but not that of PGHS-2, which is consistent with the known difference in efficiencies of activation of the two PGHS isoforms by hydroperoxide (48, 49, 55, 56), with PGHS-1 requiring a higher concentration of hydroperoxide for activation of the resting enzyme. PGHS-2 requires much smaller amounts of hydroperoxide for catalytic activation, both because the tyrosyl radical in PGHS-2 is formed more rapidly by hydroperoxide than that of PGHS-1, and also because the tyrosyl radical of PGHS-2 persists even after the ferryloxo heme is reduced back to the resting (ferric) state, in contrast to the parallel decline of the tyrosyl radical and ferryloxo heme in PGHS-1.

It may be hypothesized that increasing the concentration of substrate would attenuate the inhibitory action of ApAP by elevating levels of PGG_2. Indeed, raising the concentration of AA has been shown to reduce the inhibitory action of ApAP (40, 57). This effect of substrate concentration on ApAP action would seem to be the consequence of the elevated level of PGG_2 formed by the increased concentration of substrate rather than a result of a competition of ApAP with AA at the PGHS-cyclooxygenase active site, because it has been reported that ApAP does not prevent the inhibition of the enzyme by either indomethacin or aspirin (40, 58). We also demonstrate that increasing PGG_2 production by elevation of the concentration of the enzyme also attenuated the action of ApAP. In addition, the action of ApAP is reversed by the addition of PGG_2 itself, providing direct evidence that it is the product of the enzyme that antagonizes the inhibitory effect of ApAP. Importantly, the potency of ApAP is enhanced markedly when low concentrations of both substrate and enzyme are used.

The concentration of exogenous AA also determines the potency of ApAP in both platelets and HUVECs. The IC_{50} for ApAP on the platelet at the lowest concentration of AA tested (0.5 μM) was similar to that observed when the platelets are activated by thrombin in these studies and by clotting of blood *ex vivo* in the investigation of Patrono (47). Thus, the effect of ApAP observed when cells are exposed to low concentrations of exogenous AA most closely approximates its action on the release of prostanoids by physiologic signaling.

Exogenous AA can serve directly as substrate for the PGHSs, and it also may cause activation of phospholipase A_2 with release of endogenous esterified AA; because platelets and HUVECs will have different levels of activated phospholipase A_2 after exposure to a given concentration of exogenous AA, they will release different amounts of AA from endogenous stores. Indeed, the explosive activation of phospholipase A_2 in platelets by either exogenous AA or receptor-dependent stimuli will lead

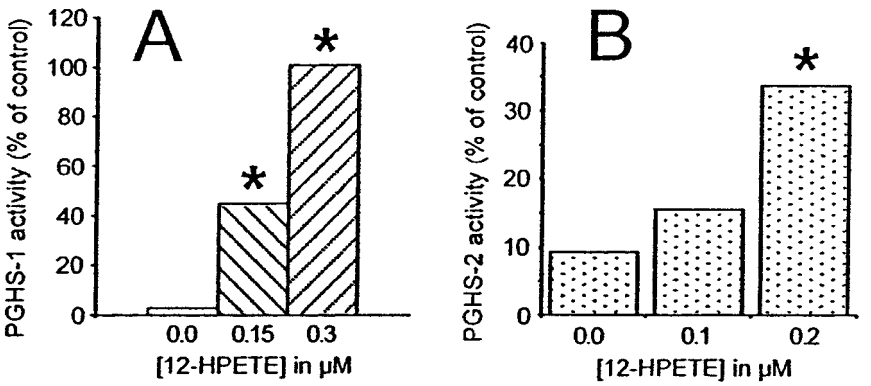

Fig. 8. Effect of 12-HPETE on the inhibition of PGHS by ApAP. PGHS-1 and PGHS-2 were reconstituted (*Material and Methods*). 12-HPETE was added at the indicated final concentrations, immediately followed by [^{14}C]AA (final concentration, 0.5 μM). The reaction was stopped after 8 sec. PGHS activity is expressed as a percentage of the control with the indicated concentration of 12-HPETE, to which no ApAP was added. (*A*) PGHS-1 (5.4 nM) was preincubated at 37°C with 1.0 mM ApAP. The data points represent the average of six values. (*B*) PGHS-2 (10 nM) was preincubated at 37°C with 1 mM ApAP. The data points represent the average of at least three values. The significance of differences between each concentration of 12-HPETE was determined (*, $P < 0.001$ when compared with the two other concentrations).

to a burst of PGG_2 formation, which may act in addition to 12-HPETE to cause the resistance of the platelet to the inhibitory action of ApAP.

Lipoxygenases also are present in key cellular contributors to the inflammatory process including the 12/15 leukocyte-type lipoxygenase and the 5-lipoxygenase in murine peritoneal macrophages (59) and the 5-lipoxygenase in human alveolar macrophages (60, 61). Our finding provides the basis for a hypothesis that the hydroperoxides formed from these lipoxygenases could attenuate the action of ApAP in such inflammatory cells and thereby account for the lack of an antiinflammatory effect of the drug.

In endothelial cells stimulated with IL-1α, when the concentration of added AA is reduced to 2 μM, the IC_{50} of ApAP is 4.3 μM. This IC_{50} is congruent with plasma concentrations associated with antipyretic efficacy in humans (1). This potent effect on endothelial cells stimulated with IL-1α is of particular interest in light of the evidence that endothelial cells are the predominant site of increased PGHS-2 in the brain during pyrexia induced by cytokines or lipopolysaccharide (21–23).

In summary, ApAP inhibits PGHSs in IL-1α-stimulated endothelial cells at concentrations relevant to its antipyretic effect. By contrast, it is more than two orders of magnitude less potent as an inhibitor of PGHS activity in platelets. Inhibition of the activity of purified PGHSs is abrogated by either added hydroperoxides or by increased formation of the hydroperoxide product of the PGHS-cyclooxygenase, PGG_2. Inhibition of PGHS activity in endothelial cells is reversed by a peroxide, and 12-HPETE, a major product of AA in platelets, reverses inhibition of purified PGHS-1 activity by ApAP. Together these findings provide support for the hypothesis that the clinical action of ApAP is mediated by inhibition of PGHS activity, and that the concentration of hydroperoxide contributes to the cellular selectivity in its action.

We thank Elizabeth Shipp and Tina Masterson for excellent technical assistance, Joseph Covington for providing the HUVECs, Brenda Crews and Dr. Sheila Timmons for their valuable advice, and Dr. Yu Shyr for his contribution to the statistical analysis of data. This work was supported in part by National Institutes of Health Grants GM 15431 and T32-GM 07569 and a grant from Merck–Frosst Canada & Company. J.A.O. is the Thomas F. Frist, Sr., Professor of Medicine.

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